

- Fusion of GFP to *Brucella groES* promoter by PCR and then moved it into *Brucella*. This construct expressed high levels of GFP in a *purEK* mutant *B. melitensis* vaccine candidate and is inducible in host macrophages.
- Fusion of recombinant *P. berghei* genes encoding MSP-1 and CSP to *groES* promoter by PCR and then moved it into *Brucella*. We have CSP and MSP1 DNA under the control of the *groE* promoter.

Example 5

We devised and refined a PCR method for the fusion of *Brucella* promoters with genes for heterologous antigens. This approach allows for the mixing and matching of promoters and genes to rapidly optimize the expression of heterologous antigens, specifically *P. berghei* and *P. falciparum* proteins, in *Brucella* vaccine carriers.

We constructed fusion plasmids for the expression of heterologous fluorescent reporter proteins EGFP, DsRed and GFP under control of the *Brucella purE* promoter and moved plasmids into *B. melitensis*. Only GFP was expressed at low level, and this was not inducible in macrophages. We constructed plasmids to express recombinant *P. berghei* antigens MSP-1 and CSP behind the *purE* promoter and moved into *Brucella*.

In a *B. melitensis* rough mutant we expressed GFP at intermediate levels behind the kan promoter on a plasmid carrying a gene complementing the rough defect of the host strain. Rough complementation on the expression plasmid served to maintain it in the bacterium inside human macrophages, since rough strains are attenuated relative to smooth in mammalian hosts. This approach will insure the maintenance of expression plasmids in live *Brucella* vaccine carriers within host cells. We fused recombinant *P. berghei* MSP-1 to the kan promoter.

We fused GFP to the *Brucella groES* promoter. This construct yielded high levels of GFP expression in a *B. melitensis purEK* vaccine strain inducible in vivo. We fused recombinant *P. berghei* MSP-1 and CSP genes to the *groES* promoter. We have CSP and MSP1 DNA under the control of the *groE* promoter.

Example 6

A repeat of the previous persistence experiment but with younger mice yielded similar results. See Figure 8. WRRP1 bearing pGSG5 again persisted for a much longer time and at vastly higher numbers than the uncomplemented strain, and again was cleared from BALB/c spleens by 8 weeks. The data were more robust here, with lower variation; we saw 100% infection in the complemented group through 2 weeks. Mean spleen loads were consistently higher and more comparable of what is characteristic for WR201, at least in the early timepoints. We also saw a single colony in the uncomplemented group at 2 weeks, interesting because we had never seen persistence beyond a week in any previous experiment.

Looking at the dissemination of the complemented strain to the organs in these mice, as shown in Figure 9, the numbers recovered from spleens exceeded the other organs, with the exception of the lungs at 3 days. Lungs and livers were also clear by eight weeks. There was low-level dissemination to inguinal lymph nodes up to two weeks. And here was a low level and transient dissemination to the male reproductive organs, gone after 1 week. Early clearance from the male reproductive organs is an aspect that distinguishes WRRP1 bearing pGSG5 from the purine auxotroph WR201, whose persistence in these organs was extended in both mice and nonhuman primates. This indicated increased attenuation is perhaps due to decay of smoothness by loss of the complementing plasmid in the host. This attenuation indicates that severely attenuated WRRP1 with its rough defect *trans* complemented in this way may be a safer alternative to WR201 and may be as effective in immunizing against *Brucella*. WR201 was our most effective vaccine to date, providing sterile immunity in nonhuman primates.

The Sequence Listing below includes the following sequences.

SEQ ID NO: 1 is a DNA sequence encoding the *wboA* (*RfbU*) gene, a mannosyltransferase.

SEQ ID NO: 2 is an amino acid sequence of the *wboA* (*RfbU*) protein.

CLAIMS

1. An immunogenic composition comprising a live *Brucella* host cell having a rough phenotype, which host cell is sufficiently attenuated that upon exposure to a mammal the host cell will not exhibit full virulence of non-attenuated *Brucella*, wherein the host cell is transformed with a recombinant DNA construct replicable in *Brucella*, which DNA construct comprises:
 - (i) a promoter recognizable by *Brucella*, and
 - (ii) a complementation DNA fragment which is operably linked to the promoter and which complements a rough-conferring mutation in the host cell, thereby effecting a smooth phenotype in the host cell
2. The immunogenic composition of claim 1, wherein the *Brucella* host cell comprises a *Brucella* DNA fragment containing a stable non-reverting deletion mutation, having the nucleotide sequence of SEQ ID NO: 1 modified to delete nucleotides from position 1067 to position 1671.
3. The immunogenic composition of claim 1, wherein the *Brucella* host cell is *Brucella melitensis*.
4. The immunogenic composition of claim 1, wherein the *Brucella* host cell is WRRP1, having ATCC accession number PTA-3753.
5. The immunogenic composition of claim 4, wherein *Brucella* host cell WRRP1 has no antibiotic resistance markers.
6. The immunogenic composition of claim 1, wherein the *Brucella* host cell is WRR51, having ATCC accession number PTA-3754.
7. The immunogenic composition of claim 6, wherein *Brucella* host cell WRR51 has no antibiotic resistance markers.

8. The immunogenic composition of claim 1, wherein the promoter is a *Brucella* promoter.

9. The immunogenic composition of claim 1, wherein the complementation DNA
5 fragment comprises the *wboA* gene.

10. The immunogenic composition of claim 9, wherein the *wboA* complementation DNA fragment encodes a peptide required for lipopolysaccharide O-sidechain synthesis.

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11. An immunogenic composition comprising a live attenuated *Brucella* host cell having a rough phenotype, which host cell is sufficiently attenuated that upon exposure to a mammal the host cell will not exhibit full virulence of non-attenuated *Brucella*, wherein the host cell is transformed with a recombinant DNA
15 construct replicable in *Brucella*, which DNA construct comprises:

(i) a DNA fragment operably linked to a first promoter recognizable by *Brucella*, and encoding a heterologous antigen; and

(ii) a complementation DNA fragment which is operably linked to a second promoter recognizable by *Brucella*, and which
20 complements a rough-conferring mutation in the host cell, thereby effecting a smooth phenotype in the host cell.

12. The immunogenic composition of claim 11, wherein the *Brucella* host cell comprises a *Brucella* DNA fragment containing a stable non-reverting deletion
25 mutation, having the nucleotide sequence of SEQ ID NO: 1 modified to delete nucleotides from position 1067 to position 1671.

13. The immunogenic composition of claim 11, wherein the *Brucella* host cell is *Brucella melitensis*.

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14. The immunogenic composition of claim 11, wherein the *Brucella* host cell is WRRP1, having ATCC accession number PTA-3753.

15. The immunogenic composition of claim 11, wherein *Brucella* host cell
5 WRRP1 has no antibiotic resistance markers.

16. The immunogenic composition of claim 11, wherein the *Brucella* host cell is WRR51, having ATCC accession number PTA-3754.

10 17. The immunogenic composition of claim 16, wherein *Brucella* host cell WRR51 has no antibiotic resistance markers.

18. The immunogenic composition of claim 11, wherein the promoter is a *Brucella* promoter.

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19. The immunogenic composition of claim 11, wherein the heterologous antigen is selected from the group consisting of anthrax antigens, *Yersinia pestis* F1 and V antigens and F1-V fusion proteins, malaria circumsporozoite and merozoite antigens, *Plasmodium berghei* antigens, *Plasmodium falsiparum* antigens,
20 *Plasmodium vivax* antigens, *Plasmodium malariae* antigens, *Francisella* antigens, staphylococcal and streptococcal enterotoxin fragment antigens; *Burkholderia* antigens, *Coxiella* antigens, *Clostridium* epsilon toxoids, botulinum toxoids, smallpox antigens, mycobacterial antigens, cancer antigens, HIV antigens, tetanus toxoids, diphtheria toxoids, pertussis toxoid, *Helicobacter* antigens, *Borrelia*
25 antigens, *Legionella* antigens, *Bartonella* antigens, vaccinia antigens, antigen-GFP fusions, tagged antigens 6his and V5, fusions of antigens to secretory signals, and genes encoding therapeutic molecules or enzymes producing therapeutic molecules.

20. The immunogenic composition of claim 19, wherein the anthrax antigen is selected from the group consisting of *Bacillus anthracis* protective antigen and inactive variants of Edema Factor and Lethal Factor.

5 21. The immunogenic composition of claim 19, wherein the malaria antigens are CSP and MSP1 antigens of *Plasmodium berghei*, *Plasmodium falciparum*, *Plasmodium vivax*, or *Plasmodium malariae*.

22. The immunogenic composition of claim 19, wherein the DNA fragment of (i)
10 encodes an enzyme synthesizes lipids and/or polysaccharides.

23. The immunogenic composition of claim 11, wherein the complementation DNA fragment comprises the *wboA* gene.

15 24. The immunogenic composition of claim 23, wherein the *wboA* complementation DNA fragment encodes a peptide required for lipopolysaccharide O-sidechain synthesis.

25. A vaccine against infection by brucellosis, comprising a live *Brucella* host
20 cell having a rough phenotype, which host cell is sufficiently attenuated that upon exposure to a mammal the host cell will not exhibit full virulence of non-attenuated *Brucella*, wherein the host cell is transformed with a recombinant DNA construct replicable in *Brucella*, which DNA construct comprises:

- (i) a promoter recognizable by *Brucella*, and
- 25 (ii) a complementation DNA fragment which is operably linked to the promoter and which complements a rough-conferring mutation in the host cell, thereby effecting a smooth phenotype in the host cell.

26. The vaccine of claim 25, wherein the *Brucella* host cell comprises a *Brucella*
30 DNA fragment containing a stable non-reverting deletion mutation, having the

nucleotide sequence of SEQ ID NO: 1 modified to delete nucleotides from position 1067 to position 1671.

27. The vaccine of claim 25, wherein the *Brucella* host cell is *Brucella*
5 *melitensis*.

28. The vaccine of claim 25, wherein the *Brucella* host cell is WRRP1, having ATCC accession number PTA-3753.

10 29. The vaccine of claim 28, wherein *Brucella* host cell WRRP1 has no antibiotic resistance markers.

30. The vaccine of claim 28, wherein the *Brucella* host cell is WRR51, having ATCC accession number PTA-3754

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31. The vaccine of claim 30, wherein *Brucella* host cell WRR51 has no antibiotic resistance markers.

32. The vaccine of claim 25, wherein the promoter is a *Brucella* promoter.

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33. The vaccine of claim 25, wherein the complementation DNA fragment comprises the *wboA* gene.

34. The vaccine of claim 33, wherein the *wboA* complementation DNA fragment
25 encodes a peptide required for lipopolysaccharide O-sidechain synthesis.

35. The immunogenic composition of claim 34, wherein when the vaccine is administered to a vaccinee, the lipopolysaccharide O-sidechain polysaccharide is produced in vivo and an antibody to the lipopolysaccharide O-sidechain
30 polysaccharide is produced by the vaccinee in response.